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Formulation development, characterization and assessment of *In-Vitro* antifungal efficacy against *Candida albicans* of diallyl disulphide liposomal gel using 3^2 factorial design

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ABSTRACT

Objective: The aim of this study was to develop the diallyl disulphide (DADS) liposomal dispersion and further the dispersion was to formulate into gel.
Methods: The DADS liposomal system was developed using thin film hydration method. 3^2 factorial design was employed to study the effect of various factors to obtain the optimized liposomal system. Further, it was converted to gel using suitable gelling agent. The optimized liposomal system and gel formulations were evaluated for various parameters. The optimized gel was evaluated for its *in-vitro* antifungal efficacy. The stability studies for gel were carried as per International conference on harmonisation (ICH) guidelines.
Results: The optimized liposomal system was having vesicle size of 208.6 nm, % entrapment was 91.7±1.16% and loading was 11.12±0.23%. % drug permeation was 61.10±0.85%. The zeta potential showed moderate stability. The optimized DADS liposomal gel showed satisfactory results. *Ex-vivo* permeation studies showed that DADS liposomal gel possesses sustained release and drug retention study proved better retention of DADS from liposomal gel than conventional gel in skin. The stability studies showed DADS liposomal gel was stable for 30 days at 25°C /60%RH, 40°C /75%RH and refrigeration (5±3°C).
Conclusion: The results from the present study show that the sustained release profile of optimized DADS liposomal gel. The retention of DADS from liposomal gel was more than from conventional gel which helps in better pharmacological activity with least side effects.

Keywords: Diallyl disulphide (DADS), liposomal gel, permeation, sustained release and antifungal activity.

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INTRODUCTION

Garlic (Allium sativum L. fam. *Alliaceae*) a natural product used world-wide for cooking, experiences an increasing interest due to its well-known bioactivities in the latest research. Epidemiological studies have shown that the enhanced dietary intake of garlic could reduce the incidence of various types of tumors such as colon, breast, lung, prostate, and stomach. The unique flavour and biological effects of garlic are generally attributed to its characteristic organosulfur components, which are released from garlic upon their processing (mincing, chewing and etc.). The γ-glutamyl-S-alk(en)yl-L-cysteines are the primary sulfur compounds in the intact garlic, which can be hydrolysed and oxidized to yield S-alkyl(en)yl-L-cysteine sulfoxide (alliin). Alliin could be transformed to allicin when chewing or cutting, which activates the enzyme allinase. Allicin is highly unstable and instantly decompose to form various oil-soluble compounds involving diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), vinyl dithiin and ajoene if conditions are appropriate. Various pharmacological actions includes, inhibit cell division, induce apoptosis, block carcinogen activation, enhance DNA repair, induce detoxifying enzymes (anticarcinogenic/ antimutagentic), inhibit microbiological growth as antibiotics antimicrobial (antifungal, antiviral, antibacterial), scavenge oxidizing

agents, induce SOD, GPx, GST, catalase (Anti-oxidant), increase pro-inflammatory cytokine release, stimulate natural killer cells (immunomodulatory), inhibit enzymes in cholesterol and fatty acid synthesis (anti-hypolipidemic), inhibit cholesterol synthesis, enhance cholesterol turnover (anti-hypocholesterolemic), inhibit angiotensin II, induce NO and H_2S, cause vasodilation (anti-hypertensive), stimulate insulin production, interfere glucose absorption (anti-diabetic), reduce trombosane formation, change platelet membrane (anti-thrombotic) and increase GSH levels by induction of GST (hepatoprotective)[1,2]. This work aimed to study the effects of various formulation parameters on the formation of DADS loaded liposomes, liposomal gel, as well as the evaluation of the characteristics of particles distribution and the DADS concentration in liposomal system. Meanwhile, antifungal activity of DADS liposomal gel in *in-vitro* against *Candida albicans* was researched.

MATERIALS AND METHODS

Materials

Diallyl disulphide (DADS) was the gift sample from Synthite Industries Ltd., Mumbai. Phosphatidylcholine (Leciva S 70) was the gift sample from VAV Life sciences, Mumbai. Phosphatidylcholine (Phospholipon 80H) samples were obtained from Lipoid, Germany. Cholesterol was purchased from Pallav Chemicals, Mumbai. Carbopol 970, 974 and 980 from Corel Pharma Pvt Ltd., Mumbai. All the other chemicals and solvents used in the experiments were of analytical grade.

Methods

Part 1: Formulation development of DADS loaded liposomes

Preparation of DADS loaded liposomes

The DADS liposomal system was prepared by thin film hydration method[3-6]. Briefly, molar solutions of various components were prepared in chloroform containing 0.01 %w/w of butylated hydroxyl toluene. These molar solutions were added to round bottom flask with suitable glass beads in appropriate molar ratio. Chloroform was evaporated under vacuum on a rotary evaporator (JSGW equipments, India) at 60 °C (above the gel-to-liquid crystalline phase transition temperature of the phospholipids) to form a thin film on the inner surface of the round bottom flask. The dry lipid film was hydrated with addition of distilled water which was at a temperature of 60 ± 2 °C. The flask was shaken for 10 mins until a homogenous dispersion was obtained. The dispersion was subjected to probe sonication (Oscar ultrasonic Co. Ltd., India) at 90 W for 10 mins and was stored at 4 °C. In case of blank liposome, the same procedure was performed without the addition of DADS.

Optimization of DADS loaded liposomes

The effect of formulation variables (lipid film thickness, volume of hydration medium, lipid composition, cholesterol percent and DADS concentration) on the responses (EE and drug release) were statistically evaluated by applying one-way ANOVA using the software package DesignExpert® version 9.0.3. (Stat-Ease Inc., Minneapolis, MN, USA). Considering the above mentioned factors the concentration of cholesterol and drug (DADS) were selected for optimization keeping volume of hydration medium, temperature, vacuum, cycle time of sonication etc. constant. It has been reported that cholesterol provides rigidity to the liposome membrane and hence variation in its concentration will ultimately result in drug leakage. The main objective of 3^2 factorial design was to optimize the DADS loaded liposomes with maximum EE and with minimum particle size distribution[7,8]. A 2-factor 3-level full factorial design (3^2) was used for the formulation and optimization of liposomes by studying the influence of the two independent variables on the responses (Y1 and Y2) i.e. minimum particle size and percentage drug entrapment. The independent variables used were cholesterol:phospholipid (Chol:PL) molar ratio (X1=A) and amount of DADS added (X2=B). Each independent variable was kept at three levels viz. low level (-1), medium level (0) and high level (+1) as mentioned in table 1. Experimental trials were carried out at all nine possible combinations as mentioned in table 2.

Table 1: Variables and their levels in full factorial design used for optimization

Levels (Code value)	Actual values		Response	
	X1 Molar ratio of Chol:PL	X2 Amount of DADS (mg)	Y1	Y2
Low (-1)	1:6	50	Particle size (nm)	% EE
Intermediate(0)	1:4	70		
High(+1)	1:2	90		

Table 2: Possible combinations of 3^2 factorial design

Runs	Batch Code		X1 Molar ratio of Chol:PL		X2 Amount of DADS (mg)	
	Leciva S70	Phospholipon 80 H	Coded	Actual	Coded	Actual
1	LF1	PF1	-1	1:6	-1	50
2	LF2	PF2	-1	1:6	0	70
3	LF3	PF3	-1	1:6	+1	90
4	LF4	PF4	0	1:4	-1	50
5	LF5	PF5	0	1:4	0	70
6	LF6	PF6	0	1:4	+1	90
7	LF7	PF7	+1	1:2	-1	50
8	LF8	PF8	+1	1:2	0	70
9	LF9	PF9	+1	1:2	+1	90

Statistical analysis of data was carried out using analysis of variance (ANOVA). The statistical analysis was conducted using Design-Expert® version 9.0.3 trial (Stat-Ease Inc., Minneapolis, MN, USA). The software performs response surface methodology (RSM) which includes the multiple regression analysis (MRA), ANOVA and statistical optimization. RSM helps to quantify the relationship between one or more measured response and the vital input factors. It helps in determination of desirable location in the design space. This space could be maximum, minimum or an area where the response is stable over a range of factor. Statistical relationship in the form of equations was obtained which shows the effect of varying A and B on the dependent variables, Y1 and Y2. In addition, contour and 3D surface plots were obtained by Design-Expert, to represent the effect of the independent variables graphically. To determine the optimal batch, the fitted mathematical model was put in use. The two equations derived i.e. for %EE (Y2) and minimum particle size (Y1) in terms of X1 (molar ratio of Chol:PL) and X2 (amount of DADS) were sequentially employed. Experimental trials were carried out in triplicates. Specific desired values of both the responses were assigned. The formulation with the desirability value closer to unity is chosen as the optimized formulation. For the present study, the criteria for optimum batch were decided as the one which shows minimum particle size and maximum EE.

Characterization and evaluation of DADS loaded liposomes

Entrapment efficiency (EE) and drug loading (DL)

The EE of DADS loaded liposomal system was determined by indirect method[9] wherein the amount of unincorporated DADS was determined. 2 ml of DADS loaded liposomal dispersion was subjected to centrifugation at 14,000 rpm for 1 hr. The supernatant (1 ml) of the dispersion was appropriately diluted with methanol and the amount of the drug present was analysed using UV-Visible spectrophotometer (Shimadzu 1800) at 207 nm against methanol as blank. EE and DL were calculated by using below mentioned formulae,

$$\%\ Entrapment\ Efficiency\ (EE) = \frac{(Wt - Wa)}{Wt} X\ 100$$

$$\%\ Drug\ Loading\ (DL) = \frac{(Wt - Wa)}{Wt - Wa + Wl} X\ 100$$

Where, Wt-total amount of DADS added to the system, Wa-amount of DADS quantified by indirect method, Wl-amount of lipid.

Vesicle size, size distribution and zeta potential

DADS liposomal dispersion was measured for vesicle size and extent of size distribution using malvern zetasizer ZS90 (Malvern Instruments, UK) at a 90 degree scattering angle using dynamic light scattering. This technique measures the diffusion of particles moving under brownian motion and converts this to size and a size distribution using the stokes-einstein relationship[10]. For light scattering measurements, the samples were measured at a fixed angle of 90° at 25 °C. The scattering intensity was adjusted between 100-500 kcps by appropriately diluting the liposomal dispersion with double distilled water. Zeta potential was measured using laser doppler micro-electrophoresis method by malvern zetasizer ZS90 (Malvern Instruments, UK) [10]. Zeta potential was measured by filling the samples in disposable zeta cell cuvettes and placing it in the sample chamber of ZS 90.

***In-vitro* drug permeation studies**

In-vitro drug permeation studies were carried out for both DADS loaded liposomal dispersion and conventional DADS dispersion using franz diffusion cells with an effective diffusion area of 3.4619 cm^2 and a receiver volume of 20 ml. Dialysis membrane having molecular cut-off of 12-14 kDa was used which was previously soaked in the receptor medium for 24 hrs for hydration. The Receptor chamber was filled with phosphate buffer pH 6.8 and co-solvent methanol 40 %v/v. The diffusion cells were maintained at 37 ± 0.1 °C using a re-circulating water bath and the medium in the receptor chambers were stirred continuously with a magnetic bar at 50 rpm. The membranes were initially left in the diffusion cells for 30 mins in order to facilitate hydration. 1 gm of the liposome dispersion was gently placed onto membrane surface at the donor compartment. An aliquot of 2 ml of samples were withdrawn at definite time intervals (1, 2, 3, 4, 5, 6, 7 and 8 hrs) and replaced with the same amount of receptor medium to maintain sink condition. Concentration of drug permeated was determined using UV-Visible spectrophotometer (Shimadzu 1800) at 207 nm[11].

Scanning electron microscopy (SEM)

Surface morphology of the DADS loaded liposomal dispersion was characterized by SEM[12,13]. Small amount of liposomal dispersion sample was mounted on an aluminum stub with double sided adhesive tape. The tape was firmly attached to the stub and sample was scattered carefully over its surface. The stub with the sample was then sputter coated with a thin layer of gold in HUS–5GB vacuum evaporator to make the sample conductive, and then the sample was observed in Quanta 200 ESEM at an acceleration voltage of 10 KV and processed sample was subjected to SEM analysis at various magnifications.

Part 2: Formulation development of DADS liposomal gel

The DADS loaded liposomal dispersion possesses low viscosity and hence it is inconvenient for topical application. Gel formulation is normally preferred because of their controlled release characteristics, improved targeting to the affected epidermis and optimal tissue compatibility. In addition, aqueous dispersions of liposomes are basically not stable for longer time. After optimization, DADS loaded liposomal system was incorporated in the gel formulation for further studies. Gelling agents like carbopol grades 971, 974 and 980 were studied in varying concentrations that ranges from 0.5 % to 2 %w/w. Gelling agent was dispersed in distilled water by stirring using a magnetic bead at 100 rpm for 60 mins on the magnetic shaker. Propylene glycol was added in the above solution and then triethanolamine was added in a drop wise manner with continuous stirring to form a transparent gel. Mixing was continued until a transparent gel appeared, while the amount of triethanolamine was adjusted to achieve a gel with pH 7.0[14]. DADS loaded liposomal dispersion of 10 ml contains DADS equivalent to 90 mg of which 6.6 ml containing 60 mg of DADS was blended homogenously with 3.4 gm of gel which was previously prepared by dissolving carbopol 980 containing optimum concentration of gelling agent. The blending of DADS loaded liposomal dispersion and gel base made final formulation of 10 gm liposomal gel. For developing the optimized gel formulation various gelling agents and their concentrations were screened as mentioned in table 3.

Table 3: Screening and selection for optimum gelling agent grade and its concentration

Screen of gelling agents			
Gelling agent	**Grade**	**Concentration (%w/w)**	**Consistency**
Carbopol	971	0.5, 1.0, 2.0	Air entrapment
	974		Less viscous, string formation
	980		Smooth and viscous

Screening of gelling agent concentrations			
Composition	**%w/w**		
Formulation	**LG01**	**LG02**	**LG03**
Carbopol 980	0.50	1.00	2.00
Propylene glycol	1.00	1.00	1.00
Methyl paraben	0.18	0.18	0.18
Propyl paraben	0.02	0.02	0.02
Triethanolamine	q.s	q.s	q.s
Purified water	8.30	7.80	6.80

Evaluation of DADS liposomal gel

Appearance, consistency and pH

DADS liposomal gel was evaluated for its appearance by visual inspection and examined for their consistency. For pH, 1 gm of DADS liposomal gel sample was mixed with 9 ml of purified water and the pH of the resulting mixture was determined with a glass electrode instrument.

Extrudability

Collapsible aluminum tubes were filled with DADS liposomal gel formulation and crimped. The gel was extruded by applying pressure from the crimped end towards the mouth. The gel thus extruded was evaluated and graded.

Viscosity

The viscosity of the DADS liposomal gel was determined by brookfield digital viscometer, using spindle S96 (T shaped spindle) rotated at 2 rpm. The viscosity was measured at 2 rpm after 30 secs at 25 °C.

Spreadability

Glass slab apparatus was used to determine the spreadability of DADS liposomal gel formulation. The apparatus consists of two glass plates (25 × 5 cm). Lower side of plate is marked with circle. 1 gm of DADS liposomal gel sample was placed in the centre of the circle. Place slowly another plain glass plate on the marked glass plate. The gel will spread between upper and lower glass plates according to its spreading capacity. Apply pressure of 2 kg weight on the upper plate for half an hour. By using scale measure the diameter of the spread gel on glass plate and note down the average of three readings. The diameter of the gel was taken as a measure of spreadability.

Percent drug content

1 gm of DADS liposomal gel formulation (equivalent to 6 mg of DADS) was accurately weighed and dissolved in 20 ml of methanol by subjecting it to bath sonication for 30 mins. The resulting solution was then filtered through membrane filter. 1 ml of this filtrate is diluted with solvent mixture of methanol:water (5.5:4.5) to make 10 ppm of drug solution. Absorbance was measured at wavelength 282 nm using UV-Visible spectrophotometer (Shimadzu 1800) and was compared with placebo gel which was dissolved and diluted to the same volume as liposomal gel formulation and considered as blank.

***Ex-vivo* permeation studies**

Ex-vivo skin permeation studies[15] were performed using franz diffusion cells with an effective diffusion area of 3.4619 cm^2 and a receiver volume of 20 ml. After depilation and washing, goat abdominal skin was excised, thoroughly washed with the phosphate buffer, pH 6.8 solution, dried and carefully cleaned of subcutaneous fat, by wiping 3 to 4 times with a wet cotton swab soaked in methanol and then preserved at freezing temperature. Before using, the skin was thawed, pre-hydrated for 1 hr with the phosphate buffer, pH 6.8 solution and then mounted between the donor and receptor compartments of vertical franz-type diffusion cell with the horny stratum corneum side layer facing the donor compartment and the dermal side toward the receptor fluid. The receptor chamber is filled with methanol: phosphate buffer, pH 6.8 in the ratio of 40:60. The diffusion cells were maintained at 37 ± 0.1 °C using a re-circulating water bath and the fluid in the receptor chambers were stirred continuously with a magnetic bar at 50 rpm. The membranes were initially left in the franz diffusion cells for 30 mins in order to facilitate hydration. Care was taken to remove any bubbles between the underside of the diffusion membrane and the solution in the receiver compartment. Subsequently, 1 gm of the DADS liposomal gel sample was (6 mg) gently placed onto each membrane surface at the donor compartment. An aliquot of 2 ml of samples were withdrawn from each batch at definite time intervals (1, 2, 3, 4, 5, 6, 7, 8 hrs) and replaced with the same amount of medium to maintain sink condition. The amount of DADS permeated was monitored using UV-Visible spectrophotometer (Shimadzu 1800) at 207 nm. The study was conducted for DADS liposomal and DADS conventional gel. The permeation data analysis provides valuable information about the product behaviour in experiment since they indicate the amount of drug available for absorption. The cumulative amount of DADS permeated per unit area across excised goat skin as the function of time, steady state flux and permeability coefficient were determined. Amount of DADS permeated was calculated by dividing the value of cumulative amount of DADS released (mg) by area of diffusion (cm^2). Permeation Profile was derived by plotting a graph of the amount of DADS permeated at time t (mg/cm^2) versus time (hrs). The membrane was mounted on diffusion cell assembly with an effective diffusion area (orifice area) of 3.4618 cm^2. This was compared with the conventional gel. Following are the various permeation parameters that were calculated:

A. Flux is defined as the amount of material crossing through a unit area of barrier or membrane in unit time or the permeation rate of drug at steady state. Flux or J ss (mg/cm^2h) through the skin is calculated from the slope of the linear portion of plotted permeation profile curve.

B. Permeability coefficient (Kp) is the velocity of DADS permeation from the formulation through the membrane in cm/hr. It is calculated by dividing the flux (Jss) by initial concentration of drug (C0) in the donor compartment. It is dependent on the chemical structure of the drug, the physicochemical nature of the medium of application; and the physicochemical nature of the membrane. The unit of Kp is cm/hr.

$$Kp = \frac{Jss}{C0}$$

Determination of DADS loaded liposomal retention in Skin

The ability of vesicles to help retain the DADS within the skin layers was investigated by determining the amount of DADS retained in the skin samples employed in permeation studies[16,17]. After completion of the permeation experiment, skin mounted on the diffusion cell was removed. The remaining formulation adhering to the skin was scraped with a spatula. The formulation applied on skin surface was swabbed first with phosphate buffer, pH 6.8 and then gently dried by pressing between two tissue papers to remove any adhering formulation. The skin was then perforated and cut into small pieces. 20 ml of methanol was added to the mashed mass and mechanically shaken in a water shaker bath at 37 ºC for 1 hr for the complete extraction of the DADS. The resulting solution containing strips of membrane was filtered and strained. The filtrate was removed, suitably diluted and the drug content in filtrate was determined at 207 nm, using a UV-Visible spectrophotometer (Shimadzu 1800). This study was performed with DADS liposomal gel and conventional gel.

***In-vitro* antifungal activity of DADS liposomal gel**

Inoculum was prepared by dissolving (with heating) 28 gm of prepared medium in 1000 ml distilled water and boiled to dissolve completely. The microorganisms (*Candida albicans*) were streaked under aseptic conditions, and the slants were incubated at 37 ± 1 ºC for 24 hrs. These 24 hrs cultures were used for preparation of inoculum. The suspension of the microorganisms was prepared in 10 ml of sterile water and 1 ml of this suspension was added to 100 ml of the nutrient agar medium. Cultural medium was prepared by dissolving (with heating) 16.25 gm of sabouraud dextrose agar in 250 ml of purified water. The dissolved broth is sterilized for 15 mins at 121 °C at 15 lb pressure in autoclave for about 20 mins. The agar plate was prepared by adding sterilized medium and was cooled at 40 ºC. 1 ml of inoculum per 100 ml of medium was added to the conical flask. This was shaken gently to avoid the formation of air bubbles and then transferred into petri dishes so as to obtain 6 mm thickness of medium. The medium in the plate was allowed to solidify at room temperature. The sterile borer was used to prepare 2 cups of 6 mm diameter in the medium of each petri dish. 60 mg of formulated liposomal and conventional DADS gel was placed into the bored wells of the petri-plates. The formulations are allowed to diffuse in the agar at the room temperature for an hour. The petri-plates are then incubated at 37 ± 2 °C for 48 hrs in the incubator. The antifungal activity was determined by measuring the mean diameter of zone of inhibition in mm after 48 hrs of incubation[18].

Stability studies

The developed DADS liposomal gel was subjected to stability studies for the duration of 30 days. The optimized DADS liposomal gel formulation (20 gm) was filled in lacquer coated aluminium collapsible tubes and was stored at various temperature and humidity conditions 25 ± 5 °C / 60 %RH, 40 ± 5 °C / 75 %RH and refrigeration temperature (5 ± 3 °C) as per ICH guideline[19]. The samples were tested and evaluated initially and then at 15th and 30th day from the day of commencement of the study. Various parameters were evaluated such as appearance, consistency, pH, extrudability, viscosity, spreadability, percent drug content, and permeation studies.

RESULTS AND DISCUSSION

Part 01: Formulation development of DADS loaded liposomes

Design Expert software performs RSM which helps to quantify the relationship between one or more measured responses and the vital input factors. It helps in determination of desirable location in the design space. This space could be maximum, minimum or an area where the response is constant over a range of factor.

Optimization of formulation parameters for liposomes: The selected factors such as DADS concentration and molar ratio of Chol:PL influences the responses vesicle size and % EE. Table 4 and 5 shows observed responses in 3^2 factorial design for leciva S 70 and phospholipon 80H.

Table 4: Observed response in 3^2 factorial design for leciva S 70

Runs	Formulation codes for Leciva S 70	X1 molar ratio of Chol:PL		X2 amount of DADS (mg)		Dependent variable	
		Coded	Actual	Coded	Actual	Vesicle size (nm)	% EE
1	LF1	-1	1:6	-1	50	188.70	79.62
2	LF2	-1	1:6	0	70	195.30	83.40
3	LF3	-1	1:6	+1	90	208.10	91.70
4	LF4	0	1:4	-1	50	199.81	72.80
5	LF5	0	1:4	0	70	257.70	74.90
6	LF6	0	1:4	+1	90	287.90	77.20
7	LF7	+1	1:2	-1	50	201.0	68.90
8	LF8	+1	1:2	0	70	239.20	71.60
9	LF9	+1	1:2	+1	90	273.20	73.40

Table 5: Observed response in 3^2 factorial design for phospholipon 80H

Runs	Formulation codes for Phospholipon 80H	X1 molar ratio of Chol:PL		X2 amount of DADS (mg)		Dependent variable	
		Coded	Actual	Coded	Actual	Vesicle size (nm)	% EE
1	PF1	-1	1:6	-1	50	190.50	68.90
2	PF2	-1	1:6	0	70	201.30	70.50
3	PF3	-1	1:6	+1	90	309.80	75.20
4	PF4	0	1:4	-1	50	243.50	66.70
5	PF5	0	1:4	0	70	298.70	65.30
6	PF6	0	1:4	+1	90	306.80	65.10
7	PF7	+1	1:2	-1	50	237.80	64.10
8	PF8	+1	1:2	0	70	290.80	63.80
9	PF9	+1	1:2	+1	90	321.40	62.00

From the results of optimization studies, it was found that the % EE of liposomal system prepared by phospholipon 80 H was very low. Hence. Further analysis was done for liposomes prepared using leciva S 70.

For response 1: Vesicle size

Vesicle size=285.23+20.28 × A+9.11 × B-2.60 × A × B-40.65 × A2-36.45 × B2

The equation suggested that the factor A (molar ratio of Chol:PL) and factor B (amount of DADS) have positive effects on the vesicle. Positive coefficient of A term indicates that the vesicle size decreases with decrease in molar ratio of Chol:PL. Positive coefficient of B term indicate that the vesicle size decrease with decrease in drug concentration. Hence, at low level of drug concentration and low level in molar ratio of Chol:PL, optimum vesicle size could be obtained. When the coefficient values of two independent factors are compared, the value of coefficient of molar ratio of Chol:PL was found to be higher than that of DADS Concentration and hence, molar ratio of Chol:PL was considered to be a major contributing variable for vesicle size of liposomes. Vesicle size of DADS liposome was found to be from 198.7 to 315 nm range.

Effect of Chol:PL ratio on vesicle size: Response plots as shown in figure 1 indicate that an optimum ratio of 1:6 generates particles of optimum size. Increasing or decreasing this ratio causes changes in particle Size. The formation of the lipid bilayer and its fluidity is accounted by the amount of cholesterol inserted between the phosphatidylcholine molecules. Presence of cholesterol is advantageous as it makes the bilayer sufficiently flexible and also contributes to proper release of the entrapped moiety. Decreased vesicle size was obtained with the batches having Chol:PL ratio as 1:6. This could be because; the addition of cholesterol in this ratio provides optimum rigidity to the bilayer. Decrease in vesicle size might be because increased amount of phospholipids, providing space for drug molecules to get entrapped.

Effect of DADS concentration on vesicle size: The amount of DADS concentration will influence the vesicle size. This is attributed to the lipophilicity of the DADS. When the concentration of the DADS was increasing, vesicle size also increased irrespective of the ratio of the Chol:PL.

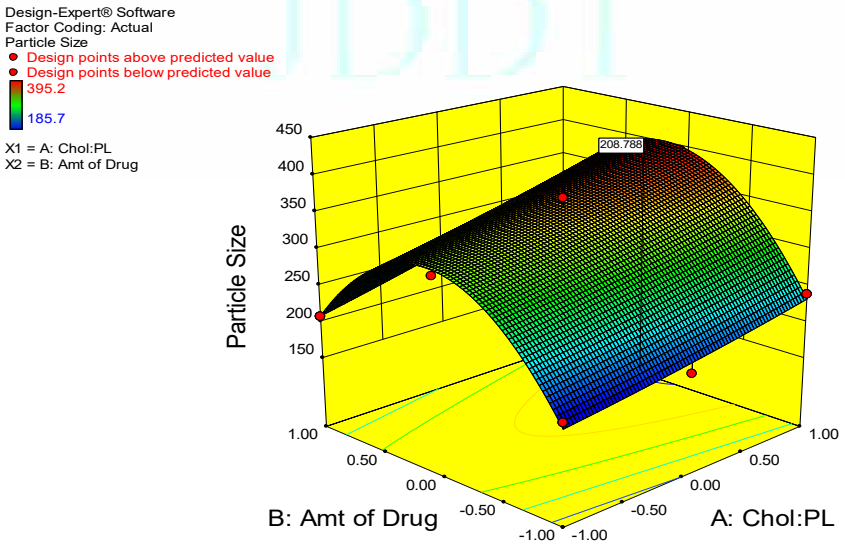

Figure 1: 3D surface response plot for the response vesicle size

The relationship between the factors and the responses was further elucidated using response surface plots. 3D response surface plots in figure 1 gives a representation of the variations in each response when the two factors are simultaneously changed from lower to higher level. It gives a three dimensional curvature of the change in response at different factor levels. It also gives the variation in design points from the predicted response value where the desired working region is represented with blue colour (minimum particle size).

For response 2: % EE

% EE=74.54-6.80 × A+3.50 × B-1.89 × A × B+3.14 × A2+0.64 × B2

The equation suggested that the factor A have a negative effect on the %EE and factor B have a positive effect on the %EE. Negative coefficient of A term indicates that the % EE increase with decrease in molar ratio of Chol:PL. Positive coefficient of B term indicates that the %EE increases with increase in DADS concentration. Hence at high level of DADS concentration and low level of molar ratio of Chol:PL, increased %EE could be obtained. When the coefficient of two independent factors are compared, the value of coefficient of molar ratio of Chol:PL was found to be higher than that of DADS concentration and hence, molar ratio of Chol:PL was considered to be a major contributing variable for %EE of liposomes. %EE of DADS liposome was found to be from 68.90 - 91.70 %.

Effect of Chol:PL ratio on %EE: Variation in the concentration of Chol:PL ratio extremely affects the EE, as illustrated in figure 2. Increase in EE at low concentration of cholesterol shows that cholesterol act as the "vesicular cement" in the molecular cavities of phospholipid bilayer and abolishes the gel to sol transition, forming less leaky vesicles. Therefore, increase in the rigidity decreases the permeability of the entrapped DADS and hence improves the EE.

Effect of DADS amount on %EE: Figure 2 suggested, increase in concentration of DADS increases EE irrespective of the ratio of the Chol:PL. This is attributed to the lipophilicity of the DADS. Therefore, EE can be increased by increasing the amount of DADS added at the optimal Chol:PL ratio.

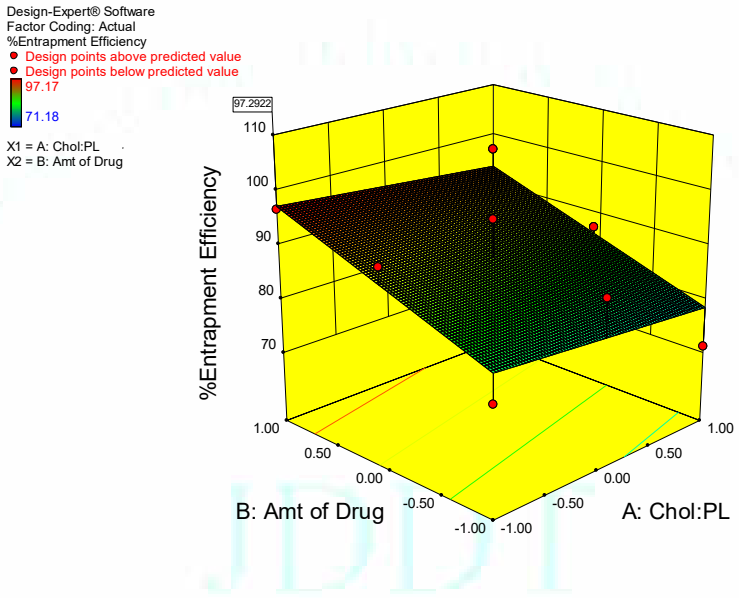

Figure 2: 3D Surface response plot for % EE of DADS

Desirability and overlay plot

The desirability plot from figure 3 suggests the region (working space) from which any formulation prepared would have results in desired range of the responses. Figure 4 representing an overlay plot obtained by the Design Expert® Software is used to determine ratio of Chol:PL and amount of DADS, so as to have minimum vesicle size and maximum %EE. From overlay plot (figure 4 and table 6), solution 1 has desirability value closer to unity and hence was the best solution. The variables associated with this solution had same values as that of batch LF3 i.e., –1 or low level of Chol: PL and +1 or high level of DADS concentration showing vesicle size 208.1 nm and % EE as 91.70 %. Hence, formulation LF3 was considered as the optimized formulation.

Table 6: Solutions for optimized batch suggested by design expert

Solution No.	Chol:PL	DADS concentration	Vesicle size (nm)	%EE
1	-1	1	240.135	90.51
2	-1	0.85	248.49	89.52
3	-1	-1	216.7	79.72
4	-1	-0.65	241.85	81.24
5	0.96	1	198.701	73.22
6	0.92	1	202.171	73.31

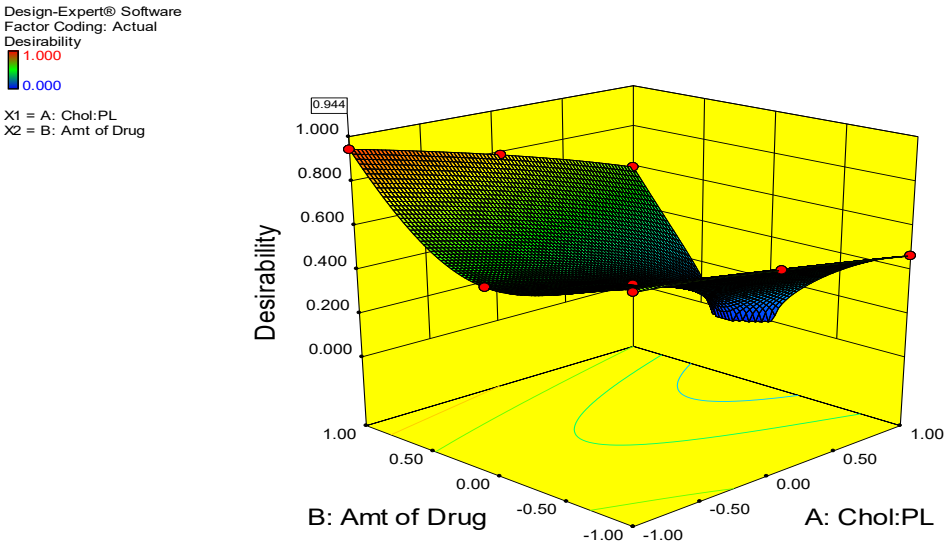

Figure 3: Desirability plot

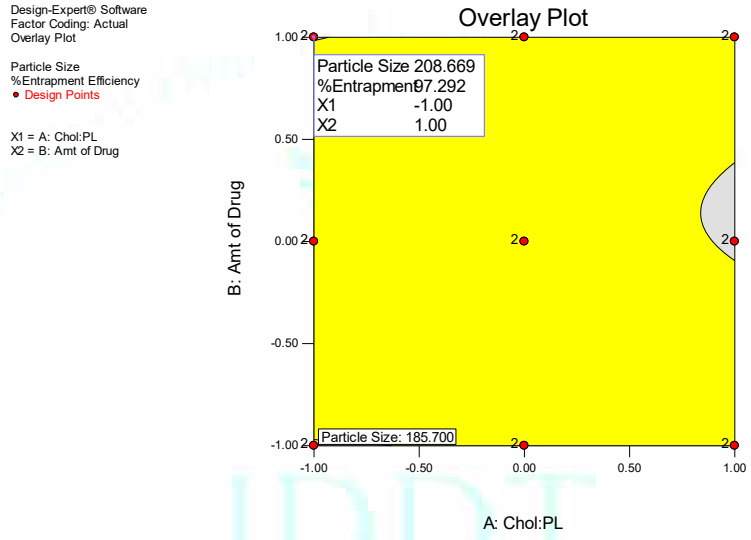

Figure 4: Overlay plot

Composition of optimized trial: Chol:PL molar ratio= 1:6 (0.1 M of Cholesterol and Phospholipid), DADS amount 90 mg and final volume of formulation is 10 ml. For the present study, the criteria for optimum batch were decided as the one which shows maximum EE and minimum vesicle size. Based on the response surface model design batches and their response graphs, 6 solutions were suggested by Design Expert® in the table 6. From the figure 3 and 4, it is clear that the desirability value of solution No. 1 i.e. corresponding to batch LF3 is closer to unity. Hence, the formulation F3 was considered the optimized formulation.

Characterization and evaluation of DADS loaded liposomes

Entrapment efficiency (EE) and Drug loading (DL)

DADS, being a lipophilic drug moiety tend to have good affinity towards the lipid bilayers. Thus, the preparation method does not have many parameters which affect the entrapment of the DADS. This was evident from the %EE which was found to be 91.7 ± 1.16 %. The drug loading was found to be 11.12 ± 0.23 %.

Vesicle size, size distribution and zeta potential

The average size was observed 208.6 nm. The liposomal dispersion showed polydispersity index 0.329. The result indicates that the liposomes were polydispersed. The 100 % of the vesicles were in the range of 208 nm size range as shown in table 7 and figure 5A. The magnitude of zeta potential gives a potential stability of the colloidal dispersion. If the particles have large positive or negative charge reveals that they repeal each other and there is dispersion stability. The liposomes in the dispersion exhibited zeta potential of -22.4 mV. Zeta potential of optimization batch is shown in figure 5B. Thus, the value of Zeta Potential of the formulation shows that liposome in the moderate stability state.

Table 7: Size distribuation of DADS loaded liposomal dispersion

Peak	Size (d.nm)	% Intensity	PDI
Peak 01	208.6	100.00	0.329

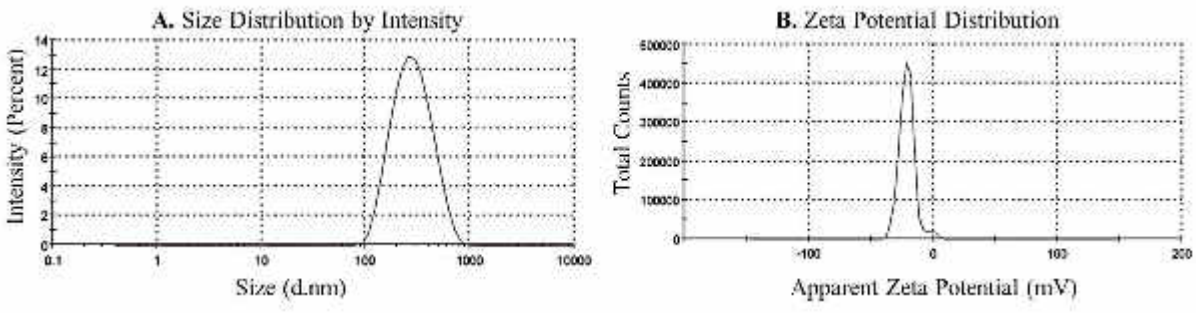

Figure 5: A. Size distribution, B. Zeta potential of DADS loaded liposomes

In-vitro drug permeation studies

The permeation of conventional DADS dispersion and DADS loaded in the liposomes were evaluated using vertical franz diffusion cells with a synthetic (cellophane) membrane between the donor and receptor compartments. The use of Franz diffusion cells provides an accurate and reliable method for evaluating active compound permeated from topical formulations. Figure 6 compares *in-vitro* permeation profile of conventional DADS dispersion and DADS loaded liposomal dispersion using mixture of phosphate buffer, pH 6.8 and methanol in the ratio 60:40 as receptor medium. It was observed that 99.50 ± 0.34 % of DADS was permeated at the end of 24 hrs from conventional dispersion whereas only 61.10 ± 0.85 % of the DADS was permeated from the liposomal dispersion. Rate and amount of DADS permeated from liposomal dispersion was lesser with initial burst release followed by sustained release. This can be due to slow diffusion of the drug through lipid matrix in the latter stage which may be advantageous, causing prolonged exposure of tumour cells to drug, increasing its clinical efficacy.

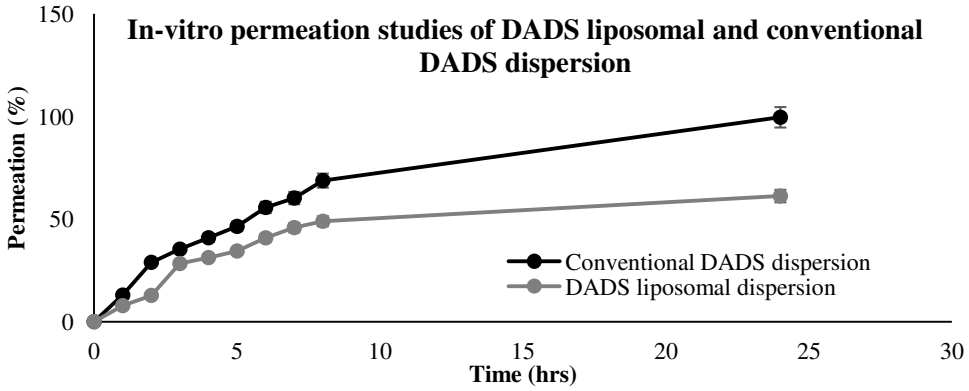

Figure 6: *In-vitro* drug permeation of DADS loaded liposomes and plain drug

Scanning Electron Microscopy (SEM)

SEM images are indicative of structure of liposome. Figure 7 indicates that developed liposomes are spherical in shape.

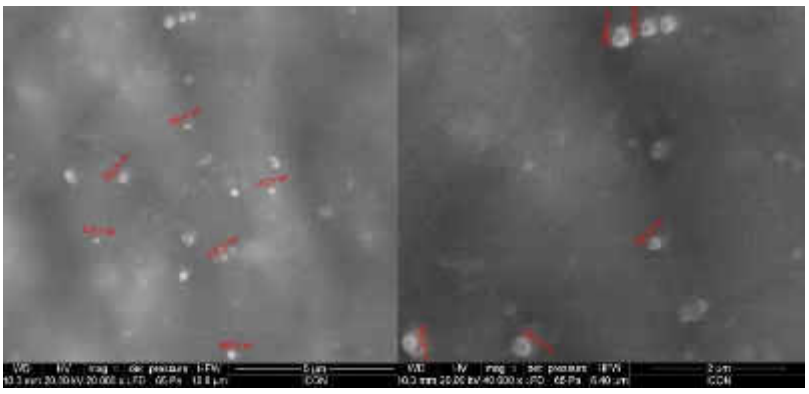

Figure 7: SEM of DADS loaded liposomes

Part 02: Formulation development of DADS liposomal gel

Carbopol gel was quite stable and had good rheological properties. The liposomal dispersion was easily incorporated into the gel without segregation or separation of the gel and liposomal dispersion. The gel prepared with carbopol 974 showed string production compared to gel prepared using other grades. The gel prepared using grade carbopol 971 exhibited air entrapment relative to gel prepared using other grades. The carbopol 980 gel possessed optimum

consistency and better incorporation potential. The results and observations of screening and selection for various gelling agents. Carbopol 980 was used as a gelling agent with propylene glycol as the humectant. Triethanolamine was used as neutralising agent for the acidic polymer. The preservative used for the formulation were methyl paraben and propyl paraben. For the further incorporation of vesicular system, carbopol 980 was selected for further studies and 0.5 %w/w to 2.0 %w/w concentration range for the polymer was studied.

Evaluation of DADS liposomal gel

Appearance, consistency and pH

The formulations prepared were translucent by nature and white in colour. All the three concentration of the carbopol 980 produced the same shade of gel. The gel was examined for its consistency and rheological properties as mentioned in table 8.

Extrudability

The gel extrudate obtained after applying pressure at the crimped end towards the cap was evaluated for its ability to form uniform cylinder without the presence of air voids. The results obtained are given in table 8.

Viscosity

Viscosities of the formulated liposomal gels using carbopol 980 were determined using brookfield Viscometer and the results were summarized in table 8.

Spreadability

The observations for spreadability of all formulations are listed in the table 8. The spreadability of the formulations is a characteristic derived from its more basic property i.e. viscosity. The greater the viscosity, the longer will be the time taken for spreading. The gels are expected to spread easily on the skin areas when applied. The spreadability also depends on the polymer in formulation, possessing typical physicochemical properties which create surface tension between slide and product.

Drug content

The drug content was found by performing the assay and the results found are shown below in the table 8. Based on the evaluation parameters, Batch LG2 was selected as the best batch giving the optimum results. This batch was further evaluated for permeation studies, drug retention studies, anti–fungal activity and stability studies.

Table 8: Evaluation parameters of various gel formulations

Carbopol 980 (%w/w)	Consistency	pH	Extrudability	Viscosity (cps)	Spreadability (gm.cm/sec)	Drug content (%)
0.50	+	6.70 ± 0.05	+	127857 ± 921	5.12 ± 0.02	95.94 ± 0.32
1.00	++	6.80 ± 0.03	++	148900 ± 586	7.78 ± 0.06	97.83 ± 0.86
2.00	+++	7.17 ± 0.05	+++	186699 ± 321	5.59 ± 0.05	93.82 ± 0.41

+=good, ++=optimum, +++=higher/harder. Date given in triplicates.

***Ex-vivo* permeation studies**

The permeation studies were carried out using goat abdominal skin. The permeation analysis was carried out to compare the permeation potentials of the DADS liposomal gel (LG2) with conventional DADS gel. Figure 8 indicates the amount of drug permeated across goat abdominal skin from the formulations. The amount of drug permeated from the DADS liposomal gel (LG2) was much slower than the conventional DADS gel. Lower flux and permeability coefficient of liposomal gel is suggestive of prolonged drug release. Liposomal gel produced sustained release of DADS because of the presence of several lipid bilayer that release the DADS slowly over prolonged period of time. The cumulative amount of DADS permeated at the end of 24 hrs, flux and permeability coefficient were calculated for each of the formulations. Comparison of drug kinetic permeation parameters obtained provides insight to sustained drug permeability in DADS liposomal gel formulation. The table 9 depicts the comparison of permeation parameters liposomal and conventional DADS gel formulations at the end of 24 hrs.

Table 9: Drug kinetic permeation parameters

Parameter	Liposomal DADS gel	Conventional DADS gel
Amount permeated (mg)	1.7001±0.0029	2.4617±0.0093
Flux (mg/cm² hr)	0.05904±0.00291	0.08862±0.00197
Permeability coefficient	0.006711±0.000075	0.008466±0.000045

Date given in triplicates.

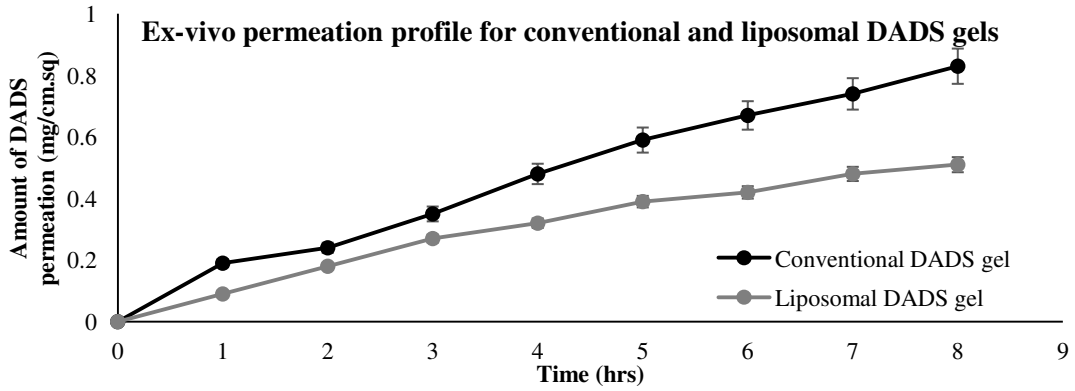

Figure 8: Permeation Profile of conventional and liposomal (LG2) DADS gels

Determination of DADS loaded liposomes retention in Skin

In–vitro skin deposition of DADS from both formulations was calculated and compared. The DADS liposomal gel and conventional DADS gels showed 15.30 ± 0.59 % and 9.11 ± 0.65 % drug retained in membrane respectively. Thus, the amount of DADS retained in membrane was more for liposomal gel than conventional gel.

***In-vitro* antifungal activity of DADS liposomal gel.**

The antifungal activity of DADS liposomal gel (LG2) was determined by measuring the diameter of inhibition zones in mm. The results for *Candida albicans* are shown in figure 9. The zone of inhibition was considerably larger for the liposomal gel (29.51 ± 3.18 mm) as compared to the conventional gel (13.82 ± 2.24 mm) at 0.60 % concentration of drug at the end of incubation.

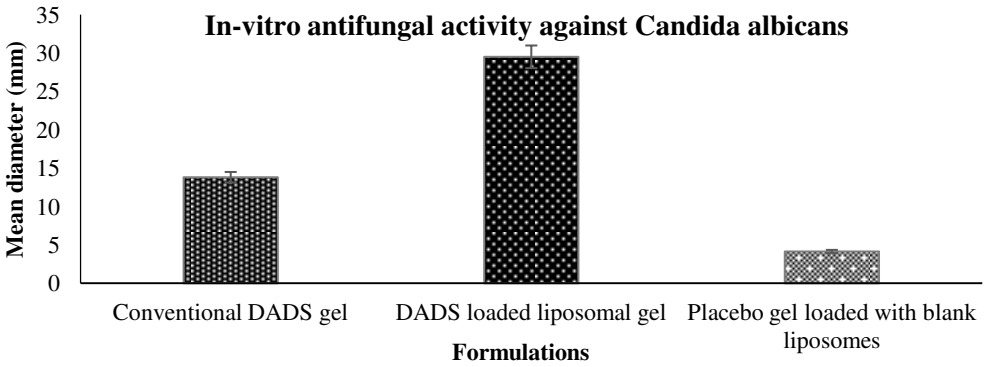

Figure 9: In-vitro antifungal activity against *candida albicans*

Stability studies

The stability studies of the DADS liposomal gel were carried out by subjecting the formulation to three conditions, at 25 °C / 60 %RH, 40 °C / 75 %RH and refrigeration temperature (5 ± 3 °C). The samples were analysed at predetermined time points for various parameters mentioned below. All the parameters were in the acceptable limits (table 10, 11 and 12) which showed that formulations were stable over the period of 30 days. There was no significant increase in the pH, drug content, viscosity and spreadability. Also the appearance was similar as that observed initially. The permeation analysis data of the formulation mentioned in the figure 10 suggested a constant release pattern of the drug thereby indicating the stability of liposomal gel. It was observed that the formulation was more stable when kept in refrigerated conditions (5 ± 3 °C) as compared to 25 °C / 60 %RH, 40 °C / 75 %RH. Although the difference was not substantial. All the parameters were in the acceptable limits which showed that the formulation was stable over a period of 30 days.

Table 10: Evaluation parameters of DADS liposomal gel at 25 °C /60 %RH

Parameter	0 day	15 days	30 days
Appearance	Translucent white	Translucent white	Translucent white
Extrudability	++	++	++
Spreadability	5.78 ± 0.32	5.56 ± 0.47	5.47 ± 0.64
pH	6.80 ± 0.18	6.70 ± 0.15	6.65 ± 0.22
Viscosity (cps)	147012 ± 243	146919 ± 146	146901 ± 212
Drug content (%)	97.70 ± 0.16	97.30 ± 0.11	97.10 ± 0.54

+=good, ++=optimum, +++=higher/harder. Date given in triplicates.

Table 11: Evaluation parameters of DADS liposomal gel at 40 °C / 75 %RH

Parameter	0 day	15 days	30 days
Appearance	Translucent white	Translucent white	Translucent white
Extrudability	++	++	+
Spreadability	5.78 ± 0.32	5.62 ± 0.55	5.587 ± 0.34
pH	6.80 ± 0.18	6.78 ± 0.06	6.64 ± 0.08
Viscosity (cps)	147012 ± 243	146953 ± 476	146683 ± 458
Drug content (%)	97.70 ± 0.16	97.10 ± 0.78	96.70 ± 0.55

+=good, ++=optimum, +++=higher/harder. Date given in triplicates.

Table 12: Evaluation parameters of DADS liposomal gel at refrigeration (5 ± 3 °C)

Parameter	0 day	15 days	30 days
Appearance	Translucent white	Translucent white	Translucent white
Extrudability	++	++	++
Spreadability	5.78 ± 0.32	5.72 ± 0.09	5.72 ± 0.15
pH	6.80 ± 0.18	6.8 ± 0.08	6.70 ± 0.09
Viscosity (cps)	147012 ± 243	147141 ± 248	147100 ± 258
Drug content (%)	97.70 ± 0.16	97.42 ± 0.09	97.38 ± 0.18

+=good, ++=optimum, +++=higher/harder. Date given in triplicates.

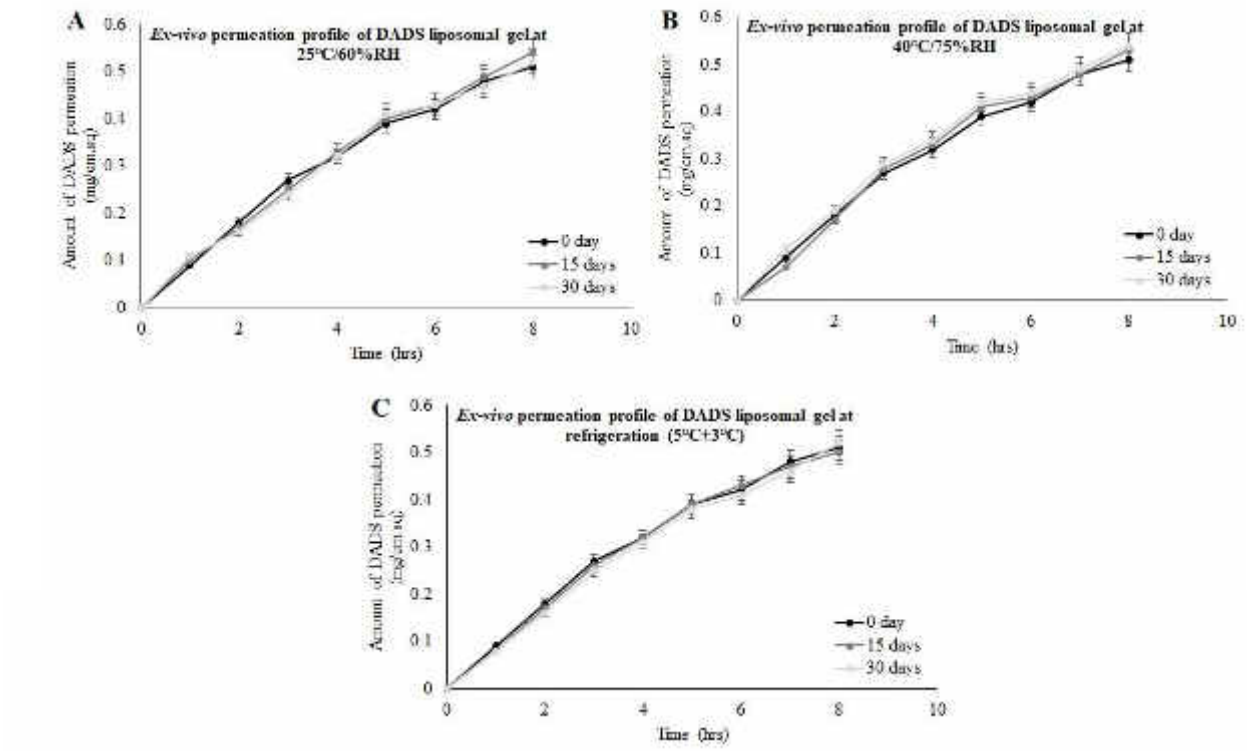

Figure 10: Permeation study of DADS liposomal gel at A. 40 ℃/75 %RH, B. 25 ℃/ 60 %RH and C. refrigeration temperature (5 ± 3 ℃).

Over the last several years, the frequency of life-threatening fungal infections has increased dramatically, particularly among cancer, diabetic and immunocompromised patients. The yeast *Candida albicans* is an opportunistic pathogen that causes life-threatening disease in immunocompromised mammalian hosts. In fact, candidiasis has been found to account for approx. 52–63 % of all nosocomial fungal infections. The high occurrence of candidiasis has been attributed, at least in part, to selective pressure induced by the increased use of antifungal agents[18]. Most of the available antifungal agents are plagued by dose limiting toxicity issues as well as other harmful effects. To make matters worse, many antifungal drugs are losing their effectiveness because of the development of drug resistance in many pathogens. This consequently demands an increasing dose requirement, which results in higher chances of toxic manifestations. A potential and increasingly widely accepted way to circumvent this problem is to turn to plant-based alternative therapeutics over conventional drug therapy that show improved antimicrobial activity and reduced toxicity. Among various plant products, garlic has been traditionally used in diet and in medicinal form for its anti-infective properties[20-22]. A reported study showed that the antimicrobial activity of DADS is more than the other garlic components[23,24]. The improved efficacy of liposome encapsulated DADS can be correlated to preferential delivery to pathogen-harbouring macrophages, while the free form of DADS had inadequate access to affected sites. Particulate formulations like liposomes are specifically taken up by macrophages. The drug-loaded macrophages further act as secondary depot for the drug[18]. The antifungal activity against *candida albicans* showed higher for DADS liposomal gel than the conventional gel because of longer retention of DADS in vesicular form which helps in more pharmacological activity over a period of time. The higher retention of DADS from liposomal gel than conventional gel helps in decreasing the dose to get same pharmacological effects as that of conventional formulations. Further, reducing the dose helps in unwanted pharmacological effects. In spite of the well-established role of DADS against both bacterial and fungal pathogens, its usage could not be translated into the clinical setting mostly because of its poor solubility. Thus the poor aqueous solubility of DADS hampers the development of a suitable dosage form that can facilitate its systemic distribution upon administration into the host. The problem is likely to be circumvented through the use of an appropriate delivery system, such as liposomes, that can solubilize the drug molecule efficiently and modify the distribution of the drug in the body. It is also desirable that such a novel drug formulation should facilitate controlled release and preferential delivery at the site of infection.

CONCLUSION

The DADS liposomal system was prepared by using thin film hydration method using leciva S 70 as a vesiclular component, cholesterol as rigidity modulator and butylated hydroxyl toluene as antioxidant. The liposomal system was optimized by using 3^2 full factorial design by considering the effect of independent variables (level of ratio of vesicle forming component–Chl:PL and drug) on the dependent variables (vesicle size and %EE). It was seen that the Chol:PL ratio of 1:6 and drug concentration of 90 mg showed maximum EE and minimum vesicle size. The optimized liposomal system contains 208.6 nm vesicle size and zeta potential value of -22.4 mV which shows moderate stability of system. The system contains maximum EE of 91.7 ± 1.16 % and loading was found to be 11.12 ± 0.23 %. The *in-vitro* permeation studies showed that 99.50 ± 0.34 % of DADS was permeated at the end of 24 hrs from conventional dispersion whereas only 61.10 ± 0.85 % from liposomal system confirming sustained profile of DADS. In order to improve the stability of the liposomal system, it was incorporated into gel formulation. The optimized gel formulation possessing the satisfactory parameters. The *ex-vivo* permeation studies and drug retention studies proved longer and more pharmacological effects of DADS liposomal gel than the

conventional gel. This may result in retention of drug in skin layer and it will not permeate to greater extent through the skin thus avoiding systemic side effects. The stability studies proved that the formulation was stable over a period of 30 days as per the various storage conditions mentioned by ICH.

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